γ-H2AX as protein biomarker for radiation exposure

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Summary. For large scale exposures of the human population to ionising radiation, there is a need for cost-effective high throughput assessment of radiation exposure levels from biological samples to allow triage decisions to be made. Here we discuss the usefulness of the DNA damage marker γ-H2AX for this purpose. Foci of γ-H2AX form in response to radiation-induced DNA double-strand breaks and can be quantified by immunofluorescence microscopy or flow cytometry. Several studies have analysed this marker in patients' blood samples to determine radiation exposures during various diagnostic or therapeutic radiation treatments. Such planned exposures involve only a moderate number of samples which can be obtained at a prearranged time following exposure. In contrast, application of this method as a triage tool in large scale radiological emergencies demands high throughput sample processing and analysis. The rapid kinetics of γ-H2AX induction and loss presents a major challenge to its successful application as a triage tool. These and other as yet unresolved questions are discussed.

Key words: γ-H2AX, double-stranded DNA break, triage, biological dosimetry, radiation accident.

Riassunto *(γ-H2AX come biomarcatore dell'esposizione a radiazioni)*. In presenza di un'esposizione a radiazioni ionizzanti che colpisce la popolazione umana su larga scala è necessaria una valutazione di massa economicamente efficace dei livelli di esposizione a radiazioni effettuata in base a campioni biologici, al fine di prendere decisioni nel triage di pronto soccorso. In questo articolo discutiamo l'utilità in tali circostanze del marcatore del danno al DNA, γ-H2AX. γ-H2AX foci si formano come risposta delle rotture del doppio filamento del DNA indotte da radiazioni ionizzanti e possono essere quantificate tramite microscopia immunofluorescente o citometria a flusso. Svariati studi hanno già analizzato questo marcatore su campioni di sangue dei pazienti al fine di determinare l'esposizione alle radiazioni nel corso di trattamenti radiologici di tipo diagnostico o terapeutico. Queste esposizioni pianificate coinvolgono solo un piccolo numero di campioni che può essere ottenuto in momenti prestabiliti dopo l'esposizione. Invece, l'applicazione di questo metodo come strumento di triage in caso di emergenze ad ampia scala richiede un numero grande di campioni e analisi. L'elevata cinetica dell'induzione e perdita di γ-H2AX presenta difficoltà rilevanti per una sua applicazione soddisfacente come strumento di triage. Queste e altre questioni ancora non risolte nella disciplina vengono qui discusse.

Parole chiave: γ-H2AX, rotture del doppio filamento del DNA, triage, dosimetria biologica, incidente con radiazioni.

DNA DOUBLE-STRAND BREAKS AND H2AX PHOSPHORYLATION

DNA repair is a vitally important biological mechanism that recognises and corrects damage or abnormalities to the genome. A DNA double-strand break (DSB) is a highly cytotoxic form of DNA damage which, if not correctly repaired, can initiate genomic instability, chromosome aberrations and mutations and may eventually lead to cancer [1, 2]. DSBs are induced linearly with radiation dose, with a yield of approximately 20-40 per cell nucleus and per Gy of X- or γ-rays. Apart from ionising radiation, only very few other environmentally relevant agents or processes are known to induce significant levels of DSBs. Endogenous sources of DSBs include free radicals produced during oxidative metabolism, programmed genome rearrangements like V(D)J recombination or meiotic recombination, telomere shortening in senescent cells and accidental stoppage and collapse of replication forks during S phase. Whilst a considerable number of DSBs are thought to occur during each round of replication, spontaneous DSB levels are overall very low, especially in stationary phase primary cells.

The nucleosomal core histone variant H2AX forms part of the cellular DNA damage response. Exposure to ionising radiation triggers the large scale activation of specific DNA damage signalling and repair mechanisms. This includes the phosphorylation of H2AX in

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the vicinity of a DSB [3, 4]. Foci of phospho-H2AX (γ-H2AX) form over large chromatin domains surrounding DSBs [5]. Phosphorylation of H2AX is mediated by the phosphoinositide 3-kinase-related kinase (PIKK) family members ATM and DNA-PK in a redundant manner following ionising irradiation [6, 7] whereas ATR and, to a lesser extent, DNA-PK appear to be involved in γ-H2AX formation at sites of replication-associated breaks [8]. The formation and loss of γ-H2AX foci has been measured following exposure to radiation doses as low as 1 mGy, and foci yields have been shown to increase linearly with dose [9]. Moreover, the initial number of γ-H2AX foci formed per cell nucleus following ionising irradiation agrees with the yield of induced DSBs [5, 9, 10]. Foci disappearance over time follows DSB rejoining in repair-competent cells and a compromised function of DSB repair proteins like DNA ligase IV, DNA-PK, the MRE11-Rad50-NBS1 complex, Artemis, 53BP1 and ATM is associated with defective DSB rejoining and γ-H2AX foci loss [5, 9, 11-13]. Together, these results suggest a close one-to-one relationship between initial as well as residual radiation-induced DSBs and γ-H2AX foci.

BIOLOGICAL FUNCTION OF H2AX

H2AX is part of the H2A histone family and accounts for 2% (lymphocytes, HeLa cells) – 20% (SF268 human glioma cells) of the total H2A complement in human cells [14]. Loss of one H2AX allele in $p53^{-/-}$ mice causes a dramatic increase in the onset of tumours, suggesting that DNA damage responses may be critically affected by the expression level of H2AX [15, 16]. Cells derived from H2AX knock-out mice are radiosensitive and show a reduced capacity to repair DSBs.

The following functions have been suggested for H2AX:

- concentration of DNA damage signalling and repair proteins at DSBs (reviewed in [17]);
- signal amplification and transduction to enhance the sensitivity of the DNA damage-induced G2 cell cycle checkpoint [18];
- implementation of an Artemis-dependent pathway required for the processing of a subset of radiation-induced DSBs [19];
- recruitment of cohesin to promote sister chromatid-dependent recombinational repair (summarised in [20]);
- chromatin remodelling to assist DSB processing (reviewed in [21]);
- a chromatin anchor to prevent dissociation of break ends and enhance repair fidelity [22, 23].

SUITABLE BIOLOGICAL MATERIAL FOR γ-H2AX-BASED DOSIMETRY

Only a few minimally invasive procedures are available to obtain cells for biological dosimetry. Buccal cells can be collected either by scraping the patient's inner cheek with a swab or by use of mouthwash rinses. These fast, painless procedures are ideally suited for large scale sample collection. However, buccal swabs collect only a very limited number of cells for analysis. Also, all collected cells originate from one anatomical site which may rule out the use of this approach in cases of suspected partial body exposure. Most importantly, buccal cells are much more directly exposed to environmental, nutritional and life-style factors than other cell types. They appear to have a very high background levels of DNA strand breaks [24] and γ-H2AX foci (C. Arrichiello, University of Naples; personal communication) which may severely limit their usefulness for biological dosimetry.

Venous blood samples are commonly used to obtain lymphocytes for chromosome dosimetry [25]. Only about 2% of all lymphocytes are present in the peripheral blood. The others reside in other tissues, especially in the thymus, lymph nodes, tonsils, intestines, spleen and bone marrow. Eighty percent of all lymphocytes migrate between these tissues and the peripheral blood, with an overall recirculation time of about 12 hours [25]. It is estimated that the average time that a given lymphocyte is present in the peripheral blood is 30 min during which it travels through the body with a velocity of up to several dozen cm per second. These characteristics imply that blood samples taken within minutes or more than 12 hours after radiation exposure reflect the average dose given to the peripheral blood or whole lymphocyte pool, respectively, and, following partial body exposure, may contain mixed populations of irradiated and unirradiated lymphocytes. Following whole body exposure to sparsely ionising radiation, DSBs and γ-H2AX foci are randomly distributed and can be described using Poisson statistics. Mathematical tools established for chromosome dosimetry (reviewed in [25]) can be applied to γ-H2AX foci distributions to detect overdispersion, indicative of partial body exposures, and to quantify the irradiated fraction [26]. This has, however, only been shown for samples taken within half an hour after exposure. It is not yet clear whether the distribution of foci following whole body irradiation remains random at longer post exposure times.

Peripheral blood mononuclear cells and lymphocytes have recently been used in several studies of γ-H2AX formation in patients' blood samples following diagnostic or therapeutic radiation exposure [26-30]. Whilst several millilitres of blood are taken typically, lower volumes should provide enough material for γ-H2AX analysis. In fact, it may be possible to optimise the methodology for finger-prick blood samples. This simpler and faster method would be much better suited for large scale sampling in a major radiation emergency.

FOCI SCORING USING FLUORESCENCE MICROSCOPY

The scoring of foci is currently the most sensitive method for γ-H2AX analysis. A single DSB results

in the phosphorylation of thousands of H2AX proteins over chromatin domains of several megabases of DNA either side of the break. γ-H2AX foci become microscopically visible within minutes after irradiation, with an average early size of 0.2 μm^2 indicating the rapid phosphorylation of thousands γ-H2AX molecules in domains of approximately 2 Mbp [31]. Thanks to this large scale formation of γ-H2AX, focused in a sub-micron volume, foci can be easily distinguished from a relatively homogeneous background signal so that one individual DSB can be detected.

The sensitivity of the γ-H2AX assay is limited by the variability of foci levels in untreated cells, and is therefore cell type-dependent. Unirradiated peripheral blood mononuclear cells and normal human fibroblasts in stationary phase reportedly have very low levels of 0.1 γ-H2AX foci per cell or less, with low levels of variation between different donors or cell passages [9, 26, 28]. Given that on average 0.2-0.4 foci are induced per 10 mGy per cell, the γ-H2AX foci assay is capable of detecting radiation doses down to a few mGy under these conditions [9, 26, 28]. On the other hand, background foci levels can be much higher, on the order of one or several foci per cell, and much more variable for some tumour cell lines and for actively proliferating cells. Accordingly, the smallest detectable dose for this situation can be as high as several hundred mGy. It is important to consider that these estimates are only true for samples that are processed within less than an hour after irradiation. This is perfectly feasible, and has indeed been done, for the planned radio-diagnostic or -therapeutic exposures described above. However, it is quite unrealistic to expect that blood samples can be obtained within an hour of exposure in a radiation emergency setting. With increasing post-exposure time, the number of γ-H2AX foci decreases rapidly to about 50% of the initial level within one hour *(Figure 1)*. This initial fast decline is followed by slower loss of the ~30% residual foci present a few hours after exposure. Twenty-four hours post exposure, residual foci levels are still distinctly higher than background levels, at least for doses of several hundred mGy or more. Based on these characteristics, the minimum dose required for the reliable detection of a radiation exposure can be expected to increase sharply to tens of mGy within the first couple of hours and then more slowly as more and more foci are lost post exposure. Furthermore, it is likely that the inter-individual variation of foci levels increases with post exposure time which will further compromise the sensitivity of the method.

There is considerable variation among published data for foci formation in the first hour after irradiation. While some studies have observed maximum foci levels as early as 3-10 minutes, others have reported maximum levels as late as 30 min to 1 h after exposure. Several factors may influence the early kinetics of foci formation and loss. Cell lines deficient in the DNA damage kinase ATM show delayed radiation-induced γ-H2AX foci formation [12]. Also, as mentioned above, levels of H2AX abundance have been reported to vary by a factor of up to 10 between different cell lines and the level of variation in blood cells is not known. One would therefore expect some biological variation in the number of phosphorylated H2AX molecules present at each break site. Also, technical differences can play an important role. Different immunostaining protocols and reagents may produce samples with different signal to noise ratios. Differences in the optical resolution and light efficiency of the microscope and camera used for imaging foci can selectively affect the detection of small, dim foci. Any image analysis procedure, whether by eye or based on software analysis of digital images, typically contains an intensity or size threshold below which a γ-H2AX focus would be classified as a non-specific background signal. This threshold is likely to vary between individuals when scoring by eye and between

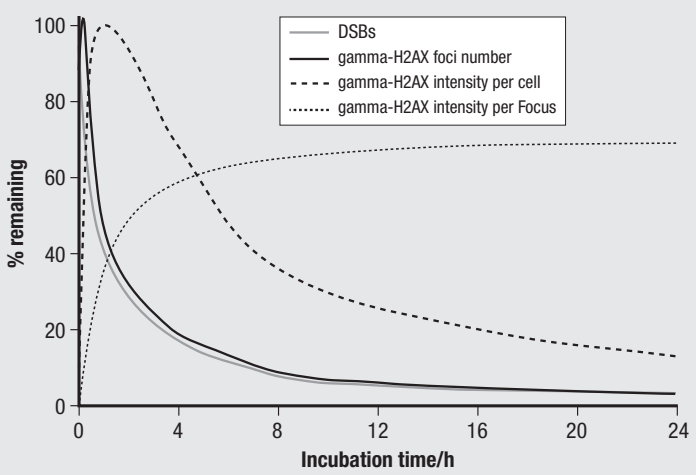

Fig. 1 | *Schematic representation of the kinetics of γ-H2AX formation and loss in relation to DSB repair following ionising irradiation. The number of γ-H2AX foci increases rapidly and reaches a maximum a few minutes after exposure. It then declines rapidly, closely following the kinetics of DSB repair. In contrast, the average γ-H2AX intensity per cell changes more slowly over time. This discrepancy can be explained by the increasing γ-H2AX intensity per focus (shown in arbitrary units) over the lifetime of the focus,* i.e. *more and more H2AX molecules in the vicinity of any unrepaired DSB are phosphorylated over time. Importantly, residual γ-H2AX levels present 24 hours after exposure still appear to be considerably higher than background levels for doses above a few hundred mGy and may therefore be used as indicator of recent exposure to a significant radiation dose.*

different software solutions and settings. All these factors are especially important at early times after exposure, when foci are still very small and dim. The increasing intensity and size of γ-H2AX foci over time simplifies their detection dramatically at later time points and makes quantitative analysis less susceptible to differences in biological characteristics, immunostaining protocols, optical properties of the microscope and thresholding introduced during image analysis.

Considering the above discussed kinetics of both foci formation and loss, the best time point for γ-H2AX analysis is probably at about 30 min to 1 h post exposure, when most of the induced foci are still present and have reached a size and intensity that allows reliable scoring. This applies obviously only to planned exposures. For radiation accidents, taking blood samples as early as that is unrealistic, and they should be taken as soon after the event as possible. Whole blood samples can be stored on ice to prevent foci loss and should be processed for γ-H2AX within about one day after the blood draw. γ-H2AX staining can be performed with whole blood smears or isolated leukocytes using standard protocols for blood processing and immunofluorescence staining. These steps typically require 3-4 hours for small numbers of samples. High throughput methods would have to be developed to enable rapid processing of large sample numbers.

The main disadvantages of foci scoring using fluorescence microscopy are the highly dynamic changes in foci numbers early after irradiation and difficulties associated with the actual scoring process which, if done by eye, can be quite time-consuming, requires some training and is somewhat subjective if slides are not coded. Several groups have developed image analysis solutions for automated foci scoring [32-35]. As foci sizes and intensities increase during repair incubation, especially in the first half hour, these software packages typically struggle to reliably quantify initial damage but seem to produce more promising results for later repair time points. In general, reliable foci scoring is limited to foci levels of less than ~20 (software) to 50 (eye) per lymphocyte, using conventional wide-field fluorescence microscopes. Overlapping foci edges in all three dimensions at higher damage levels result in "underscoring". This upper limit of foci quantification corresponds to a dose of several Gy at time points of 2 hours and beyond.

INTENSITY-BASED ANALYSIS USING FLOW CYTOMETRY

Whilst microscopic imaging and scoring of γ-H2AX foci offers the highest sensitivity, intensity-based assays for γ-H2AX are widely used in experimental research (reviewed in [36]) and may offer some advantages in terms of throughput, automation and portability. While fluorescence microscopy enables individual γ-H2AX foci and their characteristics to be imaged and analysed, flow cytometry provides a more rapid and straightforward method of γ-H2AX quantification that is based on measuring the total fluorescence intensity for each cell.

Total γ-H2AX intensity levels are dose dependent and approximately linear up to a supralethal dose of 100 Gy [37]. At 100 Gy the fluorescence signal approaches saturation due to almost total H2AX phosphorylation as the number of DSBs introduced to the genome at this dose is approximately one every 2 Mbp. Towards low doses, the sensitivity of flow cytometric analysis of γ-H2AX is not as good as that of foci scoring. The main reason is that the flow cytometer cannot distinguish specific signals (visible as foci in the microscope) from non-specific noise (homogenous background staining). This factor may limit the sensitivity of flow cytometric γ-H2AX analysis to a dose of approximately 100 mGy. In terms of triage and management or radiation casualties, this may, however, not pose a significant problem, because acute deterministic effects occur only after exposure to at least 0.5-1 Gy. For those exposed to lower doses, long term health effects such as an increased cancer risk may arise but they would not be expected to require any acute medical treatment as a result of their exposure.

As illustrated in *Figure 1*, changes in total γ-H2AX intensity over time lag behind those of foci numbers following irradiation, and appear to be less tightly coupled to DSB repair kinetics. The main reason for this discrepancy is that the number of phosphorylated H2AX molecules present at each unrepaired DSB, *i.e.* the intensity per focus, increases over time. Thanks to this effect the intensity-based assay is slightly less affected by uncertainties of the timing between exposure and blood sample collection. However, γ-H2AX intensity may well be more variable between different individuals than foci numbers. This is because any subtle difference in the activity and/or expression level of upstream kinases and phosphatases or the expression of the H2AX histone itself could have an impact on the overall levels of γ-H2AX *intensity* at all time points whereas no major effect on foci *numbers* would be expected after the first hour post exposure. Consistent with this notion, variations in γ-H2AX intensity of up to almost 2-fold in blood cells from different individuals have been reported [37].

OPEN ISSUES

In contrast to the well established cytogenetic dosimetry methods, a wide range of issues relevant to its application in biological dosimetry have yet to be addressed for the γ-H2AX assay. Some of these are listed below.

- Inter-individual variation in background levels and in the time course of γ-H2AX induction

and loss following irradiation is the most crucial issue that needs to be addressed. Without sufficient data addressing this issue, uncertainties of any dose estimates cannot be established. Residual γ-H2AX levels one or two days after exposure likely reflect individual DSB repair capacity which may be relevant to individual radiosensitivity and could potentially have some applications in individualisation of radiotherapy treatment regimes [38].

- Any recent exposure to genotoxic agents, including radio- or chemotherapy treatments and possibly tobacco smoke [39, 40], may affect the background level of γ-H2AX and may have to be taken into account when using this assay for biological dosimetry. In this context, the short-lived nature of γ-H2AX is an advantage, as only exposures that occurred within the last few days would have to be considered. Notably, trauma patients involved in a radiation accident are frequently examined by computed tomography, involving radiation doses of about 40 mSv [41]. If blood samples for γ-H2AX analysis are taken within an hour or so of such an exposure, the number of foci induced would be similar to that observed one day after a 1 Gy exposure.
- H2AX phosphorylation in the apparent absence of DSBs has been observed in cells undergoing replication, in mitosis and in XY bodies in testis. None of these should be of any relevance for studies using unstimulated lymphocytes. However, H2AX is also extensively phosphorylated during apoptosis, probably during the initiation of DNA fragmentation, and any assay system should ideally be able to distinguish between apoptotic and non-apoptotic γ-H2AX responses.
- Not much information is available about the effects of radiation type and energy on the induction and loss of γ-H2AX in lymphocytes. While γ-radiation can be regarded as the most relevant agent in the context of radiation accidents, it would be useful to have some data for neutrons and mixed neutron/γ-ray exposure.
- Further work is required to determine the effect of partial body exposure on γ-H2AX levels. *In vivo* exposures involving radiotherapy patients may help determine the effects of lymphocyte circulation through the various body organs on γ-H2AX levels in blood samples taken at different time points after treatment. Also, changes in the distribution of γ-H2AX foci over time would have to be determined.
- The impact of protracted exposures on γ-H2AX levels has not yet been fully determined. Some *in vitro* data [42] agree with the assumption that longer repair times available for DSBs induced early during protracted exposures reduce γ-H2AX levels accordingly. A further complication to consider is that for accidental protracted exposures the dose rate is likely to change considerably over time.
- It is currently unclear whether γ-H2AX analysis could also play a role in triage or biodosimetry applications for internal exposures following incorporation of radionuclides. The protracted and likely non-uniform nature of any such exposure will make any quantitative interpretation of γ-H2AX levels quite challenging. For this reason, it is unlikely that this assay can provide any reliable dose estimates. It may be able to help identify critically exposed individuals to be prioritised for chromosome dosimetry. However, urine or blood bioassays for radionuclides would probably be more appropriate in this context.
- Although several software packages have been employed for γ-H2AX foci scoring, none of these have yet been properly validated. Further development and optimisation may be required for reliable automated, unsupervised foci quantification.
- Robust high throughput procedures for blood sample collection, storage and processing both for microscopy and flow-based analysis need to be developed and validated. Required volumes should ideally be minimised to enable finger-prick blood collection.
- Implementation of the γ-H2AX assay as rapid triage tool would tremendously benefit from the availability of a portable device for γ-H2AX analysis that can be set up in a casualty-receiving hospital and provide exposure estimates for triage within a few hours.

USEFULNESS OF THE γ-H2AX ASSAY FOR RAPID TRIAGE OF RADIATION CASUALTIES

The γ-H2AX assay, as currently used in a number of labs, encompasses several of the features that one would expect from an ideal biological dosimeter. These include rapid (within a few hours) and potentially automatable processing and analysis, sensitivity to doses of a few milligrays, linear dose response across a broad dose range, ability to use unstimulated lymphocytes obtained by minimally invasive procedures, and potential to reveal partial body exposures. These features make γ-H2AX an excellent candidate as a radiation-responsive protein biomarker for rapid high throughput dosimetry for use in emergency triage. However, severe limitations associated mainly with the rapid loss of the γ-H2AX signal following irradiation have to be considered. They are likely to restrict the use of the γ-H2AX assay as a triage tool to very recent radiation exposures – less than two days before blood samples are obtained. As the yield of γ-H2AX per unit dose changes rapidly over time, dose effect curves for calibration of the assay are required for multiple time points. Also, it can only provide reliable dose estimates in acute exposure situations where the exact exposure time is known. In other cases which may involve protracted exposures or a less defined time point of acute exposure, γ-H2AX

analysis may be useful as a rapid screening tool to identify individuals with high levels of DNA damage. These could be flagged up as high priority cases for classical chromosome dosimetry which may otherwise be overwhelmed with large numbers of cases.

The fast signal loss following exposure calls for excellent integration of the assay into radiation emergency management plans. Also, manual analysis of γ-H2AX is both tedious and subjective. While several different software packages for automated γ-H2AX quantification have been developed, none of the approaches are yet fully validated. Importantly, reference data for dose response relationships as well as the level of inter-individual variation for γ-H2AX base levels and its induction following irradiation have yet to be determined for a range of time points post exposure. Other unresolved aspects include the sampling, storage and high throughput processing of blood samples and the minimisation of the required blood sample volume down to a finger-prick sample.

CONCLUSION

The rapid identification of individuals exposed to critically high radiation doses is of prime importance for initial triage and medical treatment decisions [43]. To this end, a simple, fast and high throughput assay is required to quickly triage individuals into the appropriate categories of 1) worried but well, 2) low to moderate but not acutely critical exposure and 3) those requiring urgent medical intervention to improve their chances of survival. An optimised γ-H2AX-based system may fulfil these requirements in large scale emergencies where the healthcare infrastructure could otherwise be overwhelmed with (mostly) worried-well individuals.

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